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Effects of Growth Factors in Vivo

I. Cell Ingrowth Into Porous Subcutaneous Chambers

KATHERINE H. SPRUGEL, PhD,
JOHN M. McPHERSON, PhD,
ALEXANDER W. CLOWES, MD,
and RUSSELL ROSS, PhD

From the Department of Pathology, University of Washington, Seattle, Washington; Collagen Corporation, Palo Alto, California; and the Department of Surgery, University of Washington, Seattle, Washington

Growth factors secreted by platelets and macrophages may play roles in atherogenesis and in wound repair. The multiple biologic effects of these factors are being studied extensively *in vitro*, but their roles *in vivo* are relatively unexplored. The cellular responses to platelet-derived growth factor (PDGF), transforming growth factor beta (TGFβ), basic fibroblast growth factor (bFGF), and epidermal growth factor (EGF) were examined in a wound chamber model in rats. Growth factors were emulsified in bovine dermal collagen suspensions, placed in 1 × 30-mm porous polytetrafluoroethylene tubes, inserted subcutaneously, and removed after 10 days. The presence of PDGF (400 ng), TGFβ (200 ng), or bFGF (100 ng) increased the DNA content of the chambers two- to sixfold, compared with controls. Regardless of dose, EGF (100–800 ng) did not affect the DNA content. The increases in DNA observed for PDGF, TGFβ, or bFGF resulted from accumulations of varying numbers of fibroblasts, capillaries, macrophages, and leukocytes in 10-day chambers. The addition of 250 μg/ml heparin to the collagen suspension potentiated the response to PDGF and bFGF, but not to TGFβ or EGF. The clearance of ^{125}I-labeled growth factors from the chambers was biphasic. After an initial rapid phase, the remaining growth factor was slowly cleared. The half-life of the initial phase was rapid for PDGF (12 hours) and bFGF (9 hours) and somewhat slower for TGFβ (22 hours). There was no difference in the rate of clearance between collagen and collagen/heparin matrices for any of the growth factors examined. These studies demonstrate that PDGF, bFGF, and TGFβ can induce granulation tissue development in normal animals. The similarity in cellular responses to three peptides with differing *in vitro* actions suggests that the responses observed at 10 days reflect a secondary process, possibly mediated by effector cells such as macrophages, lymphocytes, or granulocytes that are attracted into the chamber by each growth factor, rather than a direct effect of the factors themselves. (Am J Pathol 1987, 129:601–613)

GROWTH FACTORS secreted by platelets and activated macrophages have been postulated to play numerous roles in repair of tissue injury and development of vascular diseases. *In vitro,* these growth factors can stimulate cell proliferation, cell migration, and changes in extracellular matrix composition of specific target cells.[1–4] Each of these actions could be important in the process of healing wounds or in the development of proliferative vascular lesions. Whether any or all of these growth factors are deposited and act *in vivo* is not yet known. The objective of these experiments was to observe the effects of platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), type beta transforming growth factor (TGFβ), and epidermal growth factor (EGF) in the complex milieu of the intact animal.

The growth factors examined are all contained in platelets, activated monocytes, or macrophages, cell types that appear to play important roles in wound healing[5,6] and atherosclerosis.[7] Although some of the biologic activities of these factors overlap, each has a unique combination of activities and target cells that are affected. PDGF is released from platelets,[8] monocytes,[9] and macrophages[10,11] and is capable of stimu-

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Address reprint requests to Katherine H. Sprugel, PhD, Department of Pathology, SM-30, University of Washington, Seattle, WA 98195.

lating mesenchymal cell migration and proliferation (reviewed by Ross et al[1]). The basic form of FGF has been demonstrated in serum[12] and in lysates of mouse peritoneal exudate cells.[13] Basic FGF is an endothelial cell mitogen[14] and also stimulates the proliferation of fibroblasts *in vitro* (reviewed by Gospodarowicz[3]). The third growth factor examined, TGFβ, is present in platelets[15–17] and can be formed and released by monocytes and macrophages.[18] In cell culture, TGFβ tends to inhibit monolayer fibroblast proliferation and under specific conditions can stimulate cell growth in soft agar.[19,20] It is a potent chemoattractant for monocytes[21] and fibroblasts[22] and, when injected subcutaneously in neonatal mice, can result in fibroplasia.[23] Epidermal growth factor (EGF) is present in platelets[24] and may be formed by activated macrophages as well.[25] EGF stimulates epithelial cells to migrate[26] and proliferate[4] and can also stimulate fibroblast proliferation.[27]

We have studied the accumulation of cells in porous expanded polytetrafluoroethylene chambers implanted subcutaneously in rats. Chambers were filled with an emulsification of bovine collagen and one or more of the growth factors. We find that some, but not all, of these growth factors stimulate a cellular response in the lumen of the chambers and that addition of heparin to the collagen matrix enhances the responses to PDGF and bFGF.

Materials and Methods

Animals

Male Sprague–Dawley rats weighing 350–400 g were obtained from Tyler Laboratories, Bellevue, Washington. The animals were housed in the Division of Animal Medicine facility at the University of Washington. Food and water were available *ad libidum,* and a 12-hour light/dark schedule was maintained. Rats were anesthetized for all surgical procedures by sequential injections of 40 mg pentobarbital/kg intraperitoneally (Anthony Products Company, Arcadia, Calif) and 0.1 ml Innovar-Vet/kg intramuscularly (fentanyl/droperidol, Pittman-Moore, Inc., Washington Crossing, NJ).

Growth Factors

PDGF was prepared from outdated human platelets as previously described.[28] For some experiments, a partially purified PDGF preparation was used. This preparation was a pool of the side fractions collected from S200 and heparin-Sepharose columns during PDGF purification. All mitogenic activity of the crude material could be inhibited by preincubation with a polyclonal goat anti-PDGF IgG. PDGF was used at a dose of 8 μg/ml collagen suspension (approximately 400 ng/chamber). TGFβ was the generous gift of Drs. Michael Sporn and Anita Roberts, National Cancer Institute, Bethesda, Maryland. The TGFβ used was isolated from outdated human platelets.[16] TGFβ was used at a dose of 4μg/ml collagen suspension (approximately 200 ng/chamber). The basic form of fibroblast growth factor was prepared and kindly provided by Dr. Andrew Baird of the Salk Institute for Biological Studies, La Jolla, California. Basic FGF (bFGF) was isolated from bovine pituitary.[14] The dose of bFGF used routinely was 2 μg/ml collagen suspension (approximately 100 ng/chamber). Receptor grade EGF, isolated from mouse submaxillary glands, was purchased from Collaborative Research, Inc., Waltham, Massachusetts. EGF was tested at doses from 2–16 μg/ml collagen suspension (approximately 100–800 ng/chamber). The basic collagen matrix used in these experiments was pepsin-solubilized, reconstituted bovine dermal collagen dispersed in saline.[29] Final protein concentration in the chambers was 25–27 mg collagen/ml. Previous characterization of this collagen preparation showed it to be composed of greater than 95% Type I collagen, with the remainder being Type III.[29] Fibrillar collagen/heparin composites were also utilized for growth factor delivery in these experiments. Preparation and characterization of these formulations has been previously described.[30] Collagen/heparin 1 (CH1) included 300 μg heparin/ml stock collagen suspension (250 μg heparin/ml collagen emulsification in chambers). The source of the heparin was Hepar Industries, Franklin, Ohio. The formulation designated collagen/heparin 2 (CH2) consisted of collagen emulsion premixed with heparin but in a lower concentration (50 μg heparin/ml stock collagen suspension, 41.5 μg heparin/ml collagen emulsification in chambers). All collagen preparations were prepared and provided by Collagen Corporation, Palo Alto, California.

Preparation of Wound Chambers

Each chamber consisted of a 1 × 30-mm tube of expanded polyfluorotetraethylene (PTFE) with a nominal pore size of 90 μ (GORE-TEX Expanded PTFE Cell Collector Tubing, generously supplied by W. L. Gore & Associates, Inc., Flagstaff, Ariz). The chambers were filled with an emulsification of growth factors and collagen or collagen/heparin under sterile conditions, and both ends of each chamber were tied with silk suture. Each chamber contained approximately 50 μl of the collagen growth factor mixture.

With a 13-gauge needle as a trocar, four to six chambers were inserted under the abdominal skin of anesthetized rats (Figure 1). In a given rat, all chambers had the same matrix: collagen, CH1, or CH2. The chambers were removed at various times after insertion and cut into four 5-mm segments. Two alternating segments were taken for DNA assay, and the remaining two segments were fixed in methanol–Carnoy's solution for histologic evaluation.

Analysis for DNA Content and Histology

After digestion for 48 hours in 1 M NaOH, duplicate aliquots were precipitated with trichloroacetic acid and extracted with potassium acetate in ethanol, then incubated with diaminobenzoic acid and the DNA content determined fluorimetrically.[31] Methanol–Carnoy's-fixed segments were embedded in paraffin, and 5-μ sections were cut and stained with hematoxylin and eosin (H&E) for histologic evaluation.

Clearance Studies

To determine how long the growth factors were retained in the collagen-filled chambers, a separate set of experiments was performed. Trace concentrations (0.1 μg/ml) of ^{125}I-growth factor were added to the growth factor solutions prior to emulsification with collagen, CH1, or CH2. Six chambers were filled with each emulsification, and all six chambers were inserted in the same rat. The chambers were removed under ether anesthesia 1, 10, 24, 48, 120, and 240 hours after insertion. The amount of ^{125}I-growth factor in each chamber was quantified before insertion and after removal by counting the chambers in a Beckman Gamma 4000 counter. Results are expressed as "percent counts remaining," comparing the counts in each chamber after removal with its preinsertion counts. Initial counts in individual chambers ranged from 300,000 to 700,000 cpm, depending upon the growth factor used.

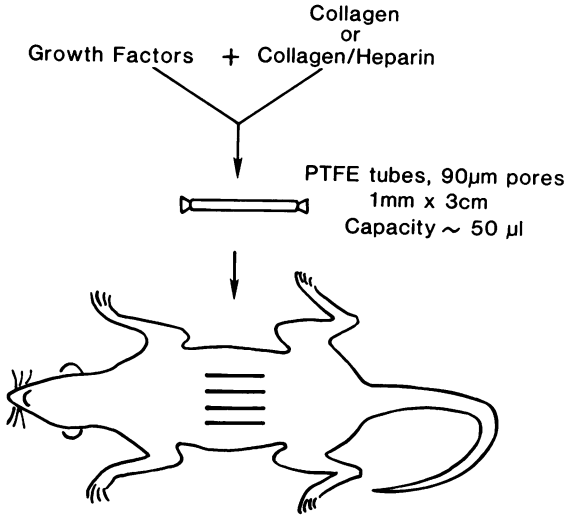

Figure 1—Schematic diagram of subcutaneous chamber model. Typically, four chambers were inserted under the abdominal skin of each rat, as shown.

Statistics

Comparisons of the effects of collagen versus collagen/heparin as a matrix for each treatment were made with the Mann–Whitney U test ($P < 0.05$). Treatment effects within the collagen or collagen/heparin groups were assessed using the Kruskal–Wallis one-way ANOVA for ranks. Specific comparisons between treatments were tested by Dunn's procedure (experimentwise, $P < 0.1$).

Results

Wound Chamber Model

After implantation in normal rats, the cellular response in subcutaneous porous chambers containing a collagen matrix or purified growth factors emulsified in the collagen matrix were evaluated for DNA content and histology at 10 days. Preliminary experiments (data not shown) were performed to establish which doses of growth factors to study in more detail and which time point to evaluate. A dose of each growth factor that resulted in a reproducible response (assessed by DNA content and histology) was selected for further study. Ten days was chosen as the end point because fibroplasia and neovascularization in response to PDGF and TGFβ were well established by 10 days but still variable at 8 days. The responses did not increase significantly if the chambers were left in the animals for 22 days.

As a control, phosphate-buffered saline containing 0.25% BSA (vehicle) was emulsified with the appropriate collagen matrix, and one chamber containing this emulsion was inserted in each rat for monitoring basal responses. Ten days after insertion, very few cells were seen in the collagen-filled lumen of vehicle-treated chambers with either the collagen or CH1 matrix (Figure 2A and B). Some cells were present in the interstices of the PTFE. The DNA content of such chambers was typically low and not affected by the addition of heparin to the matrix (Figure 3). The PTFE elicited a mild foreign-body reaction, reflected in the presence of leukocytes and giant cells at the outside margin of the PTFE. The number of these inflammatory cells was similar in all treatments, vehicle or growth factor.

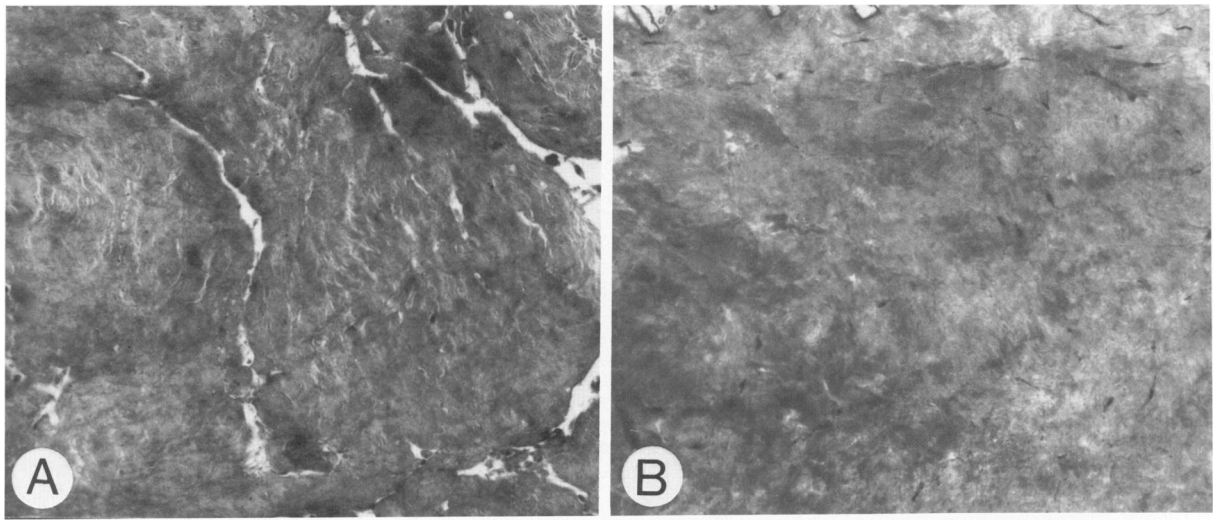

Figure 2—Light micrographs of sagittal sections of vehicle-containing chambers. (H&E, ×36) **A**—Vehicle in collagen matrix. **B**—Vehicle in collagen/heparin 1.

Response to Single Growth Factors

PDGF

The addition of partially purified and purified PDGF to the chambers resulted in a consistent increase in cells relative to vehicle controls. The DNA content of PDGF/collagen-filled chambers was 1.5–2 times vehicle, and that of PDGF/CH1-filled chambers was 3–3.5 times vehicle (Figure 3). Thus, the addition of heparin to the collagen matrix significantly enhanced the response to PDGF. As shown in Figure 4A, incorporation of PDGF in a collagen matrix increased the cells in the lumen of each chamber relative to vehicle controls. In the CH1 matrix, this increase was even more dramatic (Figure 4C). Higher magnifications of each section are presented in Figures 4B and 4D. The histologic appearance of the 10-day chambers containing PDGF resembled granulation tissue. Fibroblasts predominated, together with numerous macrophages, capillaries, and variable numbers of mononuclear cells (Figure 4B and D). Neutrophils were rare except in the lumen of the capillaries.

TGFβ

Addition of TGFβ to the collagen matrix resulted in significant increases in DNA content (Figure 3) and cell ingrowth when compared with vehicle. Use of the

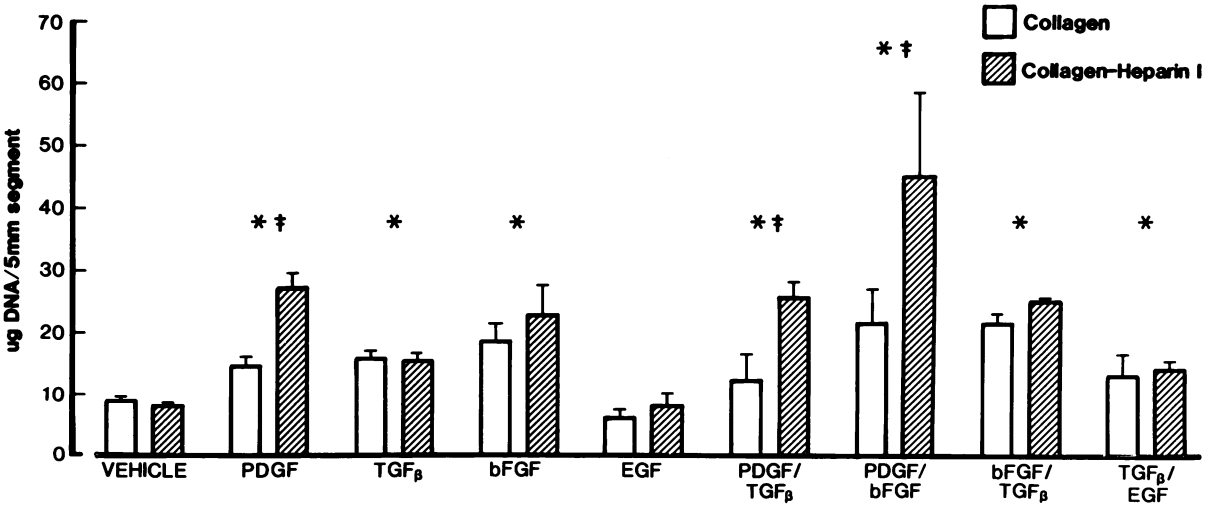

Figure 3—DNA content of chambers 10 days after insertion. Doses of growth factors were as follows: PDGF, 8 μg/ml; TGFβ, 4 μg/ml; bFGF, 2μg/ml; EGF, 8 μg/ml. n = 12–25 for vehicle and single growth factors. For combinations or EGF alone, n = 6. *Significantly different from vehicle in appropriate collagen matrix (Kruskal–Wallis one-way ANOVA, Dunn's procedure, $P < 0.05$). ‡Significant difference between collagen formulations for indicated growth factor or combination (Mann–Whitney U test, $P < 0.05$). *Bars* represent mean ± SEM.

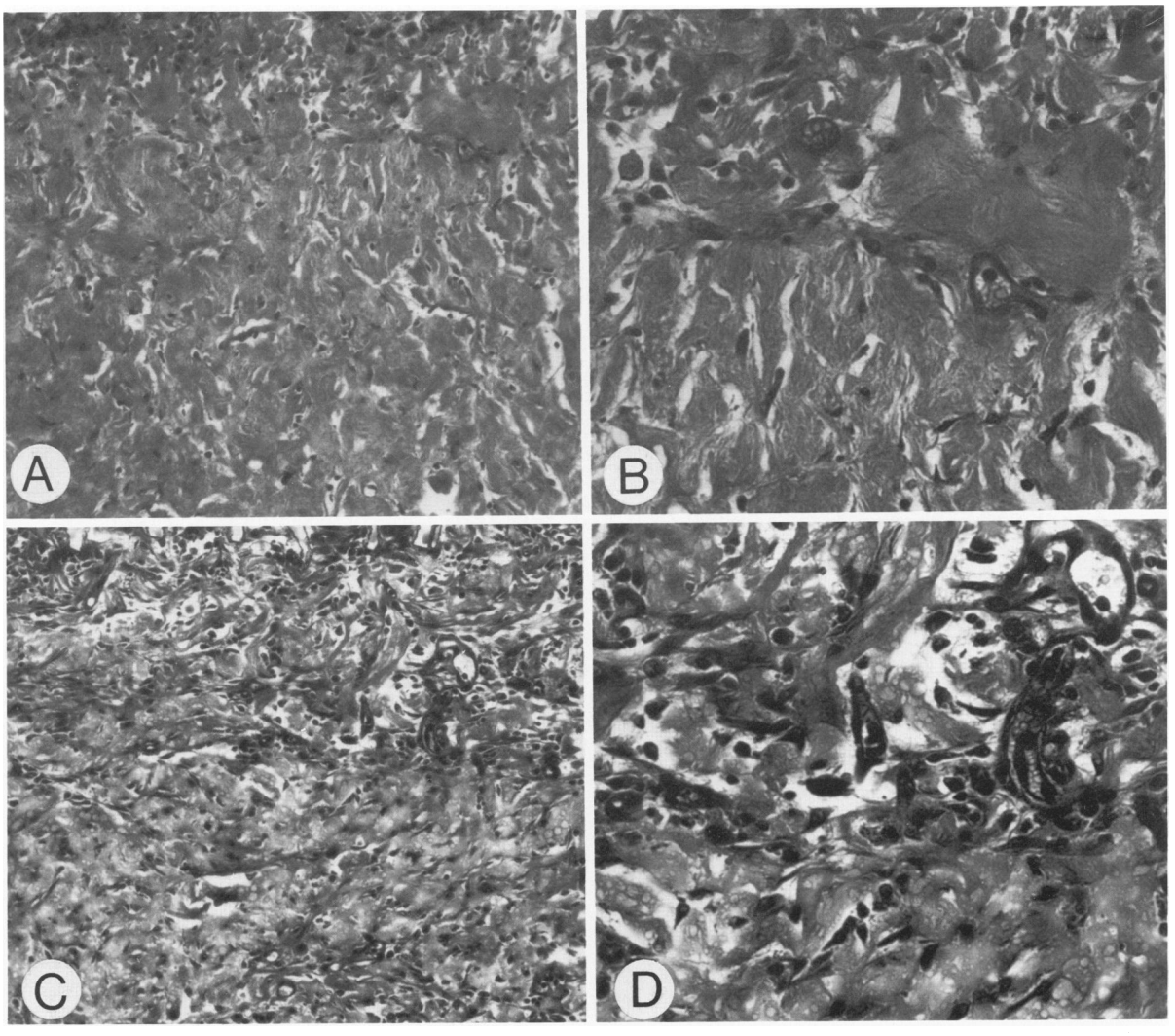

Figure 4—Light micrographs of sagittal sections of subcutaneous chambers. **A**—PDGF, 8 μg/ml, in collagen. (H&E, ×36) **B**—PDGF, 8 μg/ml, in collagen. (H&E, ×91) **C**—PDGF, 8 μg/ml, in CH1. (H&E, ×36) **D**—PDGF, 8 μg/ml, in CH1. (H&E, ×91)

collagen/heparin matrix did not enhance the response. Like PDGF and bFGF, TGFβ resulted in a granulation tissue response in the subcutaneous chambers (Figure 5A and B). In contrast to the more uniform cell distribution seen with PDGF, fibroblasts, macrophages, and capillaries tended to be clustered in units.

FGF

The responses to bFGF were more variable than those seen with the other growth factors tested. In each collagen formulation, DNA contents ranged from as low as vehicle chambers to four to seven times greater than the vehicle (Figure 3). On average, bFGF in either collagen or CH1 caused a greater cellular response than vehicle. The histologic pattern was correspondingly variable. The collagen matrix in the chambers ranged from relatively acellular to densely cellular and extremely vascularized tissue (Figure 5C and D). The capillaries were larger, dilated, and more prominent in the bFGF-containing chambers than in the PDGF- or TGFβ chambers. Regions of leukocytic infiltration were common, a response not observed with any of the other growth factors. Areas of collagen reorganization (a decrease in density of the collagen matrix reflecting resorption of bovine collagen and secretion of new collagen by the cells) were more common in bFGF-filled chambers than in those filled with PDGF or TGFβ.

A separate series of experiments was performed in an attempt to understand and control the variability of the bFGF responses. Every rat received four implanted chambers, each containing the same treatment. Chambers used for these rats were filled from a

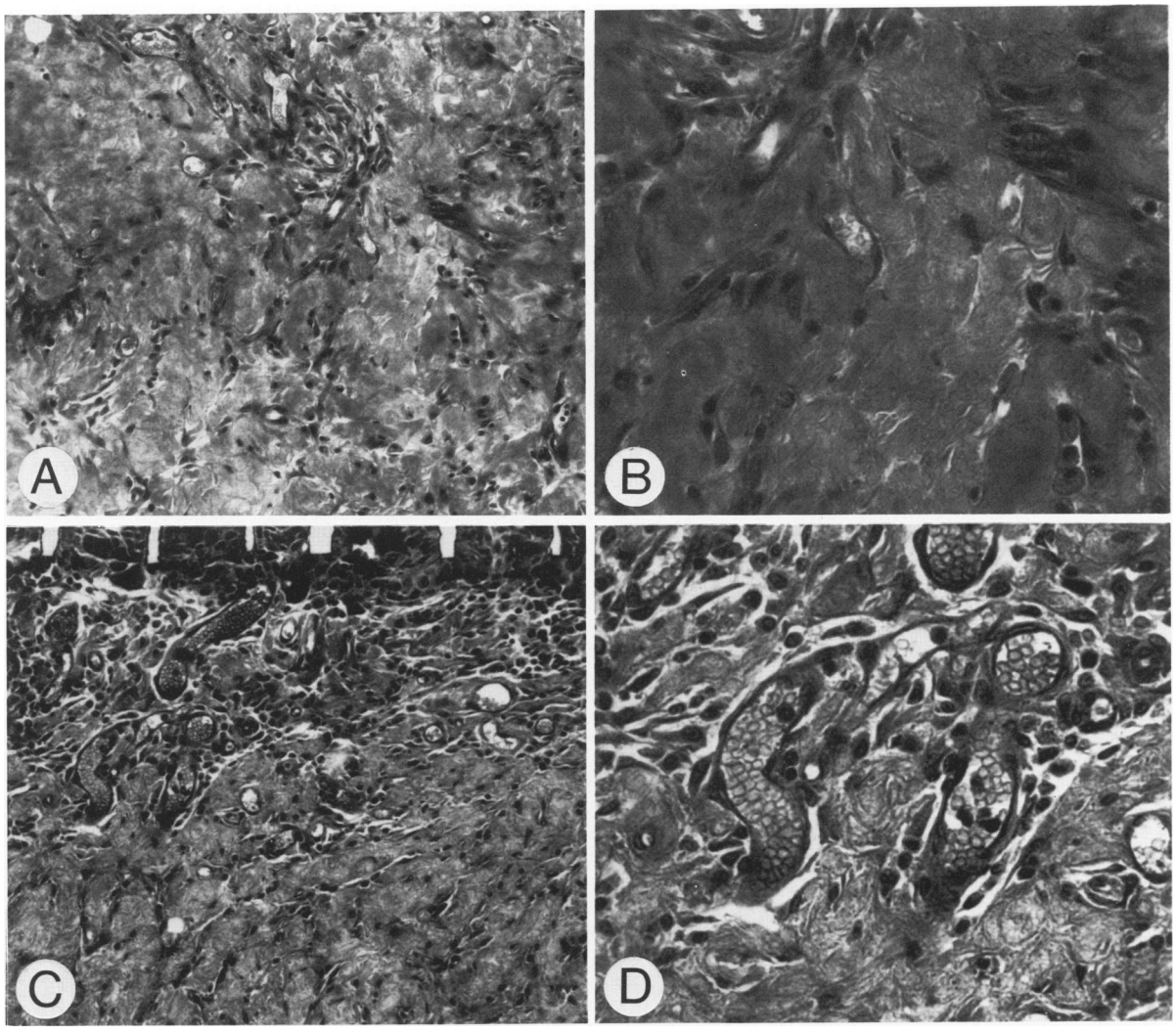

Figure 5—Light micrographs of sagittal sections of subcutaneous chambers. **A**—TGFβ, 4 μg/ml, in collagen. (H&E, ×36) **B**—TGFβ, 4 μg/ml, in collagen. (H&E, ×91) **C**—bFGF, 2 μg/ml, in collagen. (H&E, ×36) **D**—bFGF, 2 μg/ml, in collagen. (H&E, ×91)

single emulsified batch. This design allowed us to evaluate the variability in response in a given animal (ie, due to the position of the implant, lateral or medial) and the variability between animals. Additionally, a third collagen matrix was introduced into the design. In the CH1 matrix, most of the heparin was in solution and diffused rapidly out of the material when implanted.[30] The matrix designated CH2 was designed to minimize the free heparin so that most of the heparin in the material would be bound to the collagen fibrils. The rationale was that because bFGF binds to heparin, "immobilizing" (in relative terms) the heparin in the collagen matrix might help retain the bFGF, prolonging its residence in the chambers and potentially enhancing the responses or reducing the variability previously observed. The DNA contents of the chambers from these experiments are summarized in Table 1. In contrast to earlier experiments, bFGF did not enhance the responses in collagen alone when compared with vehicle. Addition of either concentration of heparin to the matrix enhanced the response. The use of a lower heparin concentration (CH2) reduced the variability somewhat but did not eliminate it. The DNA content varied by as much as twofold between chambers that were inserted in the same rats, but no specific implant position could be correlated with consistent "high" or "low" responses. It is possible that the variability of the bFGF responses observed is related to the time point studied. bFGF responses might peak earlier than 10 days and thus exhibit a wider range of responses than PDGF and TGFβ.

Table 1—Comparison of Responses to bFGF in Different Collagen Matrices*

Treatment	Collagen matrix	DNA content (μ/5 mm segment)	n
Vehicle	Collagen, CH1, CH2	10.8 (7.1–13.7)	20
2 μg/ml bFGF	Collagen	11.2 (8.2–19.6)	24
2 μg/ml bFGF	CH1	15.0 (5.0–36.6)†‡	24
2 μg/ml bFGF	CH2	19.0 (9.9–35.0)†‡§	23

*Chambers were prepared as described in "Methods," inserted subcutaneously and left for 10 days. Collagen = 25 mg/ml bovine collagen, CH1 = collagen plus 250 μg/ml heparin, CH2 = collagen plus 40 μg/ml heparin. DNA values are reported as median (range).

†Significant difference from vehicle treatment (Kruskal–Wallis one-way ANOVA, $P < 0.05$; multiple comparisons: Dunn's procedure, experimentwise, $P < 0.10$).

‡Significant difference from bFGF in collagen (Kruskal–Wallis one-way ANOVA, $P < 0.05$; multiple comparisons: Dunn's procedure, experimentwise, $P < 0.10$).

§Significant difference from bFGF in CH1 (Kruskal–Wallis one-way ANOVA, $P < 0.05$; multiple comparisons: Dunn's procedure, experimentwise, $P < 0.10$).

EGF

EGF did not stimulate a response in this model at doses ranging from 100 to 800 ng/chamber. Neither DNA content nor histology was different from the vehicle controls at any of the doses tested. DNA content for EGF alone and combinations containing EGF at doses of 400 ng/chamber are shown in Figure 3. The EFG used in these experiments was active, as indicated by its ability to compete for binding to A431 cells with ^{125}I-EGF in a radioreceptor assay (data not shown).

Responses to Combinations of Growth Factors

It is unlikely that single growth factors are released from platelets or macrophages under physiologic conditions. In platelets, for example, PDGF, TGFβ, and EGF seem to be stored in alpha granules[8,17,24] and could potentially be released simultaneously in response to the same stimulus. We therefore tested combinations of growth factors to determine whether their effects were additive, synergistic, or inhibitory. Chamber DNA contents in these experiments are summarized in Figure 3 to facilitate comparison of the effects of combinations with single growth factors. Each rat in these experiments had four chambers: vehicle, growth factor A, growth factor B, and a chamber containing both factors A and B. All chambers in a given rat had the same collagen matrix. In general, the combinations of two growth factors generated a response no different than either of the component factors alone. Figure 6A illustrates a typical response in a chamber filled with 8 μg/ml partially purified PDGF and 2 μg/ml bFGF. The combination of partially purified PDGF and bFGF showed a tendency toward synergy in individual chambers with the CH1 matrix, but the overall response was sufficiently variable that it can only be considered a trend. Histologically, the combinations reflected the characteris-

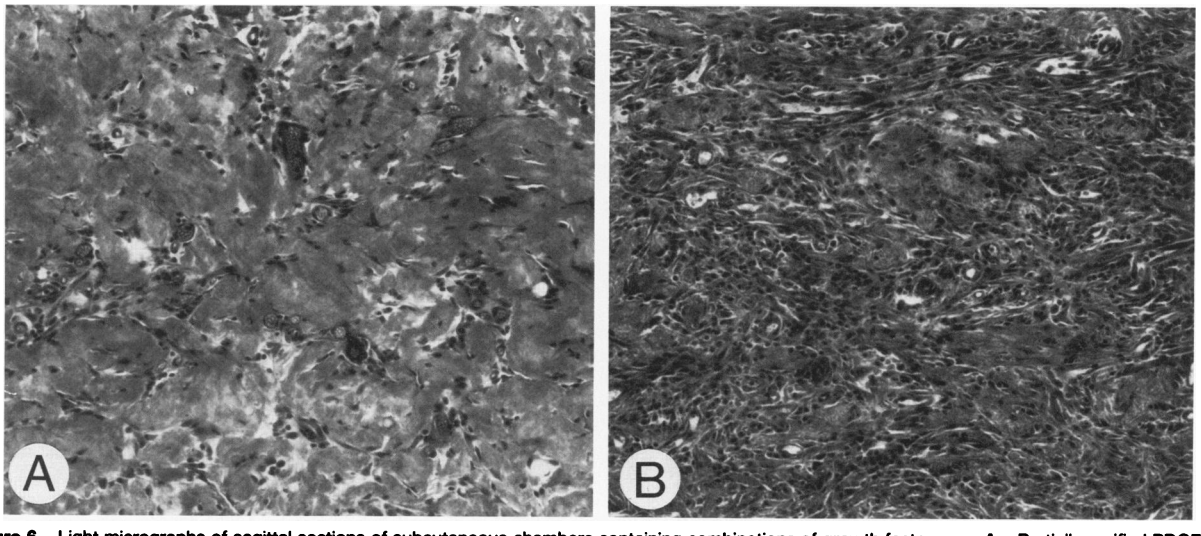

Figure 6—Light micrographs of sagittal sections of subcutaneous chambers containing combinations of growth factors. **A**—Partially purified PDGF, 8 μg/ml, plus 4 μg/ml TGFβ in CH1. **B**—Partially purified PDGF, 8 μg/ml, plus 2 μg/ml bFGF in CH1. (H&E, ×36)

tics of both factors involved. For example, the combination of PDGF and bFGF resulted in a greater fibroblast density than bFGF alone, and larger, more numerous capillaries than PDGF alone (Figure 6B).

Clearance of Growth Factors

Because two of the tested growth factors bind to heparin, we anticipated that incorporation of heparin might prolong growth factor retention in the collagen matrix and enhance the biologic response. PDGF and bFGF both bind to heparin and did produce enhanced responses in a collagen/heparin matrix when compared with a collagen matrix. For determining whether the enhanced biologic response could be attributed to longer retention of the factors in the chambers, and for obtaining kinetic information on the behavior of factors in the chambers, clearance studies were performed. Chambers were filled with an emulsion of growth factor, trace amounts of ^{125}I-labeled growth factor, and one of the collagen formulations; they were then implanted subcutaneously and removed at various times after insertion. Figures 7, 8, and 9 illustrate the results for PDGF, TGFβ, and bFGF, respectively. The clearance of each factor occurred in two phases: an initial rapid clearance (presumably diffusion of free growth factor out of the chamber) and a second slower phase (perhaps reflecting the clearance of growth factor associated with the collagen matrix or PTFE of the chamber).

^{125}I-labeled PDGF was cleared rapidly from chambers filled with either collagen or CH1 (Figure 7). The relatively low "% counts remaining" at time zero in the PDGF clearance are an artifact of the way the chambers were prepared. When we insert the chambers, some material exuded through the pores is lost in passing through the trocar. For the PDGF experiments, this meant that the counts determined before insertion were artificially high because some of those counts were lost in the insertion process. This problem was corrected in the TGFβ and bFGF clearances by passing the chambers through a trocar before determining the precount. The half-life for the initial phase of PDGF clearance was 12 hours. The only difference between the amount of PDGF remaining

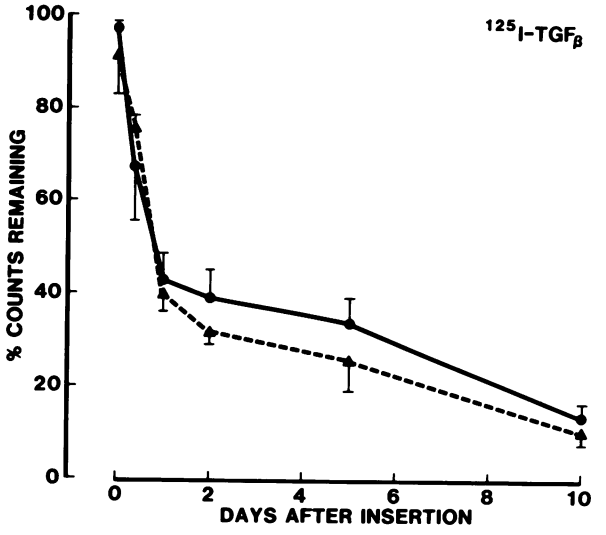

Figure 8—Clearance of ^{125}I-TGFβ from subcutaneous chambers. n = 4 for each collagen formulation. ●, collagen; ▲, CH1. *Points* represent mean ± SEM.

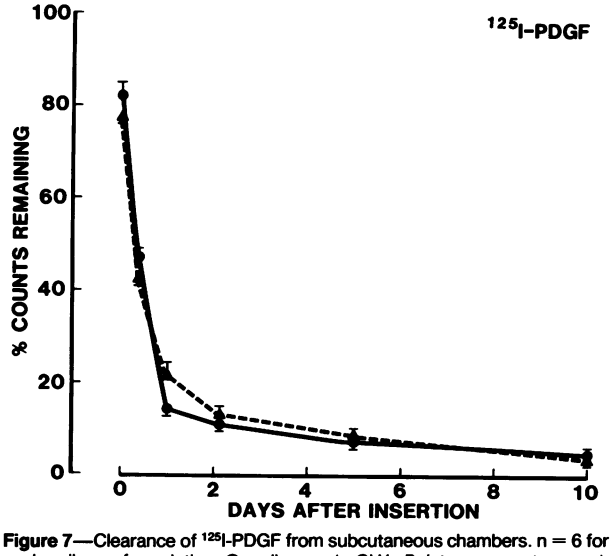

Figure 7—Clearance of ^{125}I-PDGF from subcutaneous chambers. n = 6 for each collagen formulation. ●, collagen; ▲, CH1. *Points* represent mean ± SEM.

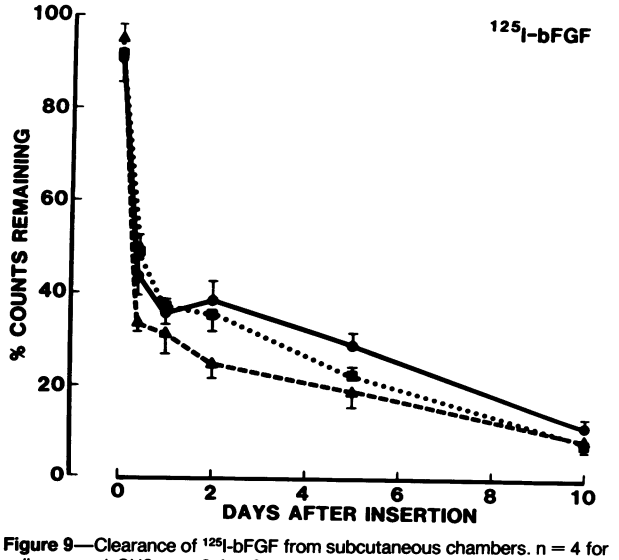

Figure 9—Clearance of ^{125}I-bFGF from subcutaneous chambers. n = 4 for collagen and CH2, n = 3 for CH1. ●, collagen; ▲, CH1; ■, CH2. *Points* represent mean ± SEM.

in collagen versus CH1 occurred at 24 hours and was relatively small. It seems unlikely that such a small and transient difference in the PDGF remaining in the chambers could account for the dramatic increase in cell ingrowth observed. ^{125}I-labeled TGFβ was cleared more slowly from the chambers than ^{125}I-labeled PDGF (Figure 8). The half-life for TGFβ was 22 hours. There was no difference in the clearance from collagen or CH1-filled chambers. The clearance of bFGF was determined in all three collagen formulations. The initial clearance was rapid and quite similar from all three matrices, with a half-life of 9 hours (Figure 9). The slower phase appeared to occur at about the same rate from each matrix, but there tended to be less ^{125}I-bFGF left in the chambers filled with CH1 than with either collagen or CH2. This may reflect the amount of material free to diffuse out of the chambers in the first phase. It is possible that the bFGF mixed with CH1 became associated primarily with the heparin molecules in the suspension, most of which were free in solution[30] and could therefore readily diffuse out. In the collagen- or CH2-filled chambers, more of the bFGF might have been associated with the collagen matrix itself, binding either directly to the collagen or to heparin bound to collagen, and thus became a part of the slower releasing pool. The differences in clearance between the collagen matrices are subtle and do not support the hypothesis that a slower clearance of bFGF in the presence of heparin is responsible for the enhanced cellular responses.

Discussion

Specific platelet- and macrophage-derived growth factors can stimulate cellular proliferation and migration *in vitro,* but their capacities to function *in vivo* are not yet defined. In this series of experiments, PDGF, bFGF, and TGFβ all produced neovascularization and fibroplasia in the chambers; EGF did not. Addition of heparin to the collagen matrix enhances the responses of the heparin-binding growth factors PDGF and bFGF, but has no effect on the TGFβ or EGF responses. The clearance of PDGF, bFGF, and TGFβ from the chambers is fairly rapid and is not altered by the addition of heparin. We anticipated that the addition of heparin might slow the clearance of PDGF and bFGF, but no differences were observed. Combining growth factors in the same chamber does not appear to alter the responses. This may be a function of the doses used. For PDGF and bFGF, these doses did not induce the maximum possible response, because addition of heparin to the matrix resulted in increased cellularity. We may not have observed the maximum possible response to TGFβ because the chambers could clearly accumulate more cells with PDGF or bFGF (compare Figures 5A and 6B). Thus, combinations of growth factors might reasonably be expected to show an additive effect. That they did not suggests that the extent of the response is limited by conditions other than the concentration of growth factor. At subthreshold doses, the growth factors might exhibit additivity or synergy. This possibility was not tested in these experiments.

Because heparin did not affect the retention of growth factors in the chambers, we considered other mechanisms by which it could enhance the cellular responses to PDGF and bFGF: alteration of the collagen fibrillar structure, direct effects of heparin on the target cells, potentiation of growth factor effects by heparin, and heparin effects on extracellular matrix modulation of cell growth. Addition of heparin to the soluble collagen preparations does have an effect on the characteristics of the matrix formed at 37 C. In the presence of heparin, the collagen fibrils are longer and form a more open lattice.[30,32] The more open meshwork may facilitate cell ingrowth, but is not sufficient to account for our observations, because all chambers filled with collagen/haparin matrix did not show enhanced responses, compared with the same growth factor in collagen alone. Chambers containing vehicle in the collagen/heparin matrix were comparable to chambers containing vehicle in collagen.

The second possibility, that heparin may have direct effects, can be ruled out by similar arguments. If heparin had an effect, for example, on directed migration of fibroblasts or endothelial cells into the chambers, we would have expected to see an enhanced cellular response in all of the chambers containing collagen/heparin. This was not observed.

The third possibility, that heparin potentiates the activity of the growth factors *in vivo,* cannot be ruled out. Some precedent for such a function exists. Heparin stabilizes and potentiates the mitogenic and chemotactic activity of the acidic form of FGF for endothelial cells[33,34] and protects both acidic and basic forms from heat or acid inactivation.[35] Additionally, heparin has been shown to potentiate the secretion of plasminogen activator from endothelial cells and the migration of endothelial cells in response to a crude 3T3-adipocyte secretion product.[36]

A fourth possibility is that heparin may alter extracellular matrix effects on cell growth. Human skin fibroblasts grown on fibrillar collagen proliferate less in response to PDGF than do the same cells grown on bare tissue culture plastic, and heparin may modify this response (J. McPherson, unpublished observations).

The responses observed at 10 days in these studies might reflect a secondary stimulus, rather than a direct effect of the growth factor originally present in the chambers. Two observations support this argument. First, three of the growth factors used produced similar cellular responses in the chambers at 10 days despite different patterns of cellular response *in vitro*. Table 2 summarizes the *in vitro* actions of each of the growth factors examined which may be relevant to wound repair. Each growth factor has a different constellation of activities and target cells that could result in protease secretion, cell migration, cell proliferation, and secretion of extracellular matrix components in the chambers. Of necessity, Table 2 is a simplification. Effects reported on cultured cells for different growth factors were not determined for each growth factor in cells from the same tissue or species. The *in vivo* responses to growth factors observed in these studies do not necessarily correlate with their *in vitro* actions. For example, chambers treated with PDGF in a collagen/heparin matrix showed extensive neovascularization. This response would not be expected to be a direct effect of PDGF, because cultured endothelial cells have no receptors for PDGF[41] and respond neither chemotactically nor mitogenically to PDGF in tissue culture. Second, the rapid initial clearance of each growth factor, particularly when compared with the pattern of cell ingrowth, suggests a secondary stimulus may be responsible for the responses observed at 10 days. Preliminary observations obtained at early time periods suggest that a classical pattern of inflammatory response occurs in these chambers. Two days after insertion, the predominant cell present is the polymorphonuclear leukocyte. By 5 days, the cell population has shifted and consists primarily of monocytes and fibroblasts. Capillary appearance and extensive fibroblast proliferation does not occur until 8–10 days after the chambers are inserted, a time when 80–95% of the growth factor is gone from the chambers.

These observations support the hypothesis shown in Figure 10. Cells may enter the subcutaneous chamber either in response to a chemotactic stimulus by the specific growth factor in the chamber or as a part of a foreign-body response to the PTFE material of which the chamber is composed, or both. Once in the chamber, the granulocytes, monocytes, and lymphocytes thus attracted could produce chemotactic and mitogenic factors that would then be responsible for the influx of fibroblasts, capillaries, and white blood cells observed in the chambers at 10 days. PDGF, bFGF, or TGFβ in the chambers might also modulate production or activation of factors secreted by these cells. This type of mechanism could account

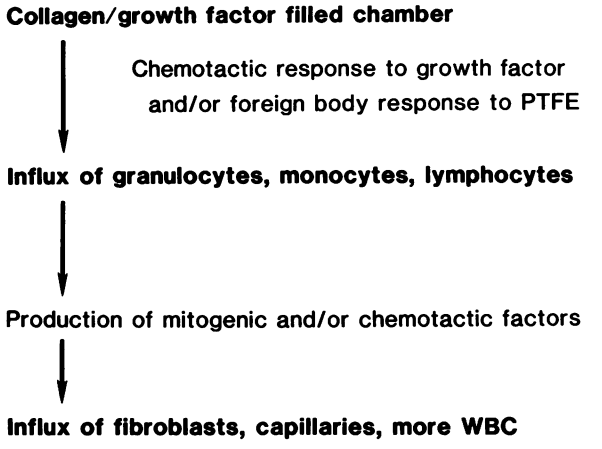

Figure 10—Hypothetical mechanism for growth factor actions in this model.

Table 2—In Vitro Actions of Selected Growth Factors*

Action	Cell†	Growth factor			
		PDGF	TGFβ	bFGF	EGF
Chemotaxis	Endos	No effect[37]	?	Stimulates‡[34]	Stimulates§
	Fibs	Stimulates[38]	Stimulates[22]	Stimulates‡[51]	No effect[38]
	Monos	Stimulates[39]	Stimulates[21]	?	?
Mitogenesis	Endos	No effect[40,41]	Inhibits[47]	Stimulates[14,52]	Stimulates[55]
	Fibs	Stimulates[42]	Stimulates[19] Inhibits[20]	Stimulates[53]	Stimulates[27]
Collagen synthesis	Fibs	Stimulates[43] No effect[23]	Stimulates[23,48]	?	?
Collagenase secretion	Fibs	Stimulates[44,45]	Stimulates[44]	Stimulates[50]	Stimulates[44,50]
Plasminogen activator activity	Endos	?	?	Stimulates[54]	?
	Fibs	No effect[46]	Stimulates[46] Decreases[49]	?	Stimulates[49]
Proteinase inhibitor secretion	Fibs	?	Stimulates[49,50]	Stimulates[50]	Stimulates[50]

*Superscript numbers refer to references cited.
†Endos, endothelial cells; Fibs, fibroblasts; Monos, monocyte/macrophages; ?, not known/reported.
‡Reported only for acidic FGF.
§Personal communication, Gary R. Grotendorst.

for the similarity of granulation tissue response to growth factors having very different *in vitro* patterns of activity.

Other investigators have also studied the effects of various growth factors in a variety of wound or wound-related models. Preparations of thrombin-stimulated platelet releasates, when incorporated in a collagen salve, have been shown to dramatically speed the healing of chronic nonhealing wounds.[56] In rats with suppressed wound healing responses, purified PDGF restored normal responses in wire mesh wound chambers, but had minimal effects in normal animals.[57,58] In these experiments, PDGF increased the rate of collagen and cell accumulation in the chambers but did not change the magnitude of the response. Cartilage-derived growth factor (probably a bFGF-type molecule[59]) produced a response similar to that we have observed with bFGF, albeit more rapid. Sponges injected with 500 ng of the cartilage-derived growth factor developed significant fibroplasia and neovascularization in 48–72 hours.[60] In a different model, eye-derived growth factor (also likely an FGF[61]) was shown to enhance the reepithelialization of burn blisters on swine epidermis.[62] TGFβ is also active in a wound healing model.[63] Wire mesh wound chambers were implanted subcutaneously in rats and repeatedly injected with crude TGFβ. An increase in the DNA, collagen, and protein content of the chambers was observed after 9 days. These experiments were repeated and extended with pure TGFβ by Lawrence and colleagues,[58] who showed that TGFβ caused an increase in cellularity, protein, and DNA content of wire mesh chambers in normal and adriamycin-treated rats. Also, direct injection of TGFβ subcutaneously in neonatal mice resulted in a profound fibroplasia and neovascularization.[23]

Unlike the other growth factors tested, EGF appears to stimulate wound healing only in wound models that involve epithelial repair; in general, higher doses and multiple applications of EGF are required to generate a response. Topical application of EGF to full-thickness skin wounds in mice increased the rate of skin closure.[64] EGF has also been shown to increase collagen synthesis in cotton-pellet–induced granulomas.[65] Incorporation of EGF in a slow-release polymer, which was then implanted subcutaneously in a sponge, increased the accumulation of DNA, protein, and cells in the sponge in the short term but did not increase the maximal response achieved.[66] Collectively these data, in conjunction with the experiments presented in this paper, suggest that therapeutic applications of growth factors may be useful in wound repair once optimal combinations, doses, and delivery modes have been characterized.

The collagen-filled, subcutaneously implanted chamber described here provides a model for the study of the mechanisms of growth factor action *in vivo*. These initial observations demonstrate that growth factors can clearly modulate cell ingrowth but suggest that the mechanisms involved are more complex than previously thought. Elucidation of the cellular events, interactions, and responses by which each growth factor contributes to the formation of granulation tissue *in vivo* may ultimately help in the design of optimal growth factor combinations for specific wound healing applications.

References

1. Ross R, Raines EW, Bowen-Pope DF: The biology of platelet-derived growth factor. Cell 1986, 46:155–169
2. Sporn MB, Roberts AB, Wakefield LM, Assoian RK: Transforming growth factor-β: biological function and chemical structure. Science 1986, 233:532–534
3. Gospodarowicz D, Neufeld G, Schweigerer L: Molecular and biological characterization of fibroblast growth factor, an angiogenic factor which also controls the proliferation and differentiation of mesoderm and neuroectoderm derived cells. Cell Differentiation 1986, 19:1–17
4. Carpenter G, Cohen S: Epidermal growth factor. Ann Rev Biochem 1979, 48:193–216
5. Ross R: The fibroblast and wound repair. Biol Rev 1968, 43:51–96
6. Leibovich SJ, Ross R: The role of the macrophage in wound repair: A study with hydrocortisone and antimacrophage serum. Am J Pathol 1975, 78:71–100
7. Ross R: The pathogenesis of atherosclerosis—an update. N Engl J Med 1986, 314:488–500
8. Kaplan KL, Broekman MJ, Chernoff A, Lesznik GR, Drillings M: Platelet α-granule proteins: studies on release and subcellular localization. Blood 1979, 53:604–618
9. Martinet Y, Bitterman PB, Mornex J-F, Grotendorst GR, Martin GR, Crystal RG: Activated human monocytes express the c-*sis* proto-oncogene and release a mediator showing PDGF-like activity. Nature 1986, 319:158–160
10. Shimokado K, Raines EW, Madtes DK, Barrett TB, Benditt EP, Ross R: A significant part of macrophage-derived growth factor consists of at least two forms of PDGF. Cell 1985, 43:277–286
11. Mornex J-F, Martinet Y, Yamauchi K, Bitterman PB, Gortendorst GR, Chytil-Weir A, Martin GR, Crystal RG: Spontaneous expression of the c-*sis* gene and release of a platelet-derived growth factorlike molecule by human alveolar macrophages. J Clin Invest 1986, 78:61–66
12. Hanai K, Kato H, Matsuhashi S, Morita H, Raines EW, Ross R: Platelet proteins, including platelet-derived growth factor, specifically depress a subset of the multiple components of the response elicited by glutathione in *Hydra*. J Cell Biol 1987, 104:1675–1681
13. Baird A, Mormede P, Bohlen P: Immunoreactive fibroblast growth factor in cells of peritoneal exudate suggests its identity with macrophage-derived growth factor. Biochem Biophys Res Commun 1985, 126:358–364
14. Bohlen P, Baird A, Esch F, Ling N, Gospodarowicz D: Isolation and partial molecular characterization of pituitary fibroblast growth factor. Proc Natl Acad Sci USA 1984, 81:5364–5368

15. Childs CB, Proper JA, Tucker RF, Moses HL: Serum contains a platelet-derived tranforming growth factor. Proc Natl Acad Sci USA 1982, 79:5312–5316
16. Assoian RK, Komoriya A, Meyers CA, Miller DM, Sporn MB: Transforming growth factor-β in human platelets: identification of a major storage site, purification, and characterization. J Biol Chem 1983, 258:7155–7160
17. Assoian RK, Sporn MB: Type β transforming growth factor in human platelets: release during platelet degranulation and action on vascular smooth muscle cells. J Cell Biol 1986, 102:1217–1223
18. Assoian RK, Fleurdelys BE, Stevenson HC, Miller PJ, Madtes DK, Raines EW, Ross R, Sporn MB: Expression and secretion of type beta transforming growth factor by activated human macrophages. Proc Natl Acad Sci USA 1987, 84:6020–6024
19. Roberts AB, Anzano MA, Wakefield LM, Roche NS, Stern DF, Sporn MB: Type β transforming growth factor: a bifunctional regulator of cellular growth. Proc Natl Acad Sci USA 1985, 82:119–123
20. Keski-Oja J, Leof EB, Lyons RM, Coffey RJ, Moses HL: Transforming growth factors and control of neoplastic cell growth. J Cell Biochem 1987, 33:95–107
21. Wahl SM, Hunt DA, Wakefield LM, McCartney-Francis N, Wahl LM, Roberts AB, Sporn MB: Transforming growth factor beta induces monocyte chemotaxis and growth factor production. Proc Natl Acad Sci USA 1987, 84:5788–5792
22. Postlethwaite AE, Keski-Oja J, Moses HL, Kang AH: Stimulation of the chemotactic migration of human fibroblasts by transforming growth factor β. J Exp Med 1987, 165:251–256
23. Roberts AB, Sporn MB, Assoian RK, Smith JM, Roche NS, Wakefield LM, Heine UI, Liotta LA, Falanga V, Kehrl JH, Fauci AS: Transforming growth factor type β: rapid induction of fibrosis and angiogenesis *in vivo* and stimulation of collagen formation *in vitro*. Proc Natl Acad Sci USA 1986, 83:4167–4171
24. Oka Y, Orth DN: Human plasma epidermal growth factor/β-urogastrone is associated with blood platelets. J Clin Invest 1983, 72:249–259
25. Madtes DK, Raines EW, Sakariassen K, Assoian RK, Sporn MB, Bell GI, Ross R: Induction of EGF and TGFα gene transcription and secretion of EGF competitive activity in activated human alveolar macrophages (abstr). J Cell Biochem 1987, Suppl 11A:33
26. Blay J, Brown KD: Epidermal growth factor promotes the chemotactic migration of cultured rat intestinal epithelial cells. J Cell Physiol 1985, 124:107–112
27. Carpenter G, Cohen S: Human epidermal growth factor and the proliferation of human fibroblasts. J Cell Physiol 1975, 88:227–238
28. Raines EW, Ross R: Platelet-derived growth factor: I. High yield purification and evidence for multiple forms. J Biol Chem 1982, 257:5154–5160
29. McPherson JM, Wallace DG, Sawamura SJ, Conti A, Condell RA, Wade S, Piez KA: Collagen fibrillogenesis *in vitro:* A characterization of fibril quality as a function of assembly conditions. Collagen Rel Res 1985, 5:119–135
30. McPherson JM, Sawamura SJ, Condell RA, Rhee W, Wallace DG: The effects of heparin on the physicochemical properties of reconstituted collagen. Collagen Rel Res (In press)
31. Clowes AW, Reidy MA, Clowes MM: Kinetics of cellular proliferation after arterial injury. I. Smooth muscle growth in the absence of endothelium. Lab Invest 1983, 49:327–333
32. Guidry C, Grinnell F: Heparin modulates the organization of hydrated collagen gels and inhibits gel contraction by fibroblasts. J Cell Biol 1987, 104:1097–1103
33. Schreiber AB, Kenney J, Kowalski WJ, Friesel R, Mehlman T, Maciag T: Interaction of endothelial cell growth factor with heparin: characterization by receptor and antibody recognition. Proc Natl Acad Sci USA 1985, 82:6138–6142
34. Terranova VP, DiFlorio R, Lyall RM, Hic S, Friesel R, Maciag T: Human endothelial cells are chemotactic to endothelial cell growth factor and haparin. J Cell Biol 1985, 101:2330–2334
35. Gospodarowicz D, Cheng J: Heparin protects basic and acidic FGF from inactivation. J Cell Physiol 1986, 128:475–484
36. Castellot JJ, Kambe AM, Dobson DE, Spiegelman BM: Heparin potentiation of 3T3-adipocyte stimulated angiogenesis: mechanisms of action on endothelial cells. J Cell Physiol 1986, 127:323–329
37. Grotendorst GR: Alteration of the chemotactic response of NIH/3T3 cells to PDGF by growth factors, transformation, and tumor promoters. Cell 1984, 36:279–285
38. Seppa H, Grotendorst G, Seppa S, Schiffmann E, Martin GR: Platelet-derived growth factor is chemotactic for fibroblasts. J Cell Biol 1982, 92:584–588
39. Deuel TF, Senior RM, Huang JS, Griffin GL: Chemotaxis of monocytes and neutrophils to platelet-derived growth factor. J Clin Invest 1982, 69:1046–1049
40. Davis PF, Ross R: Mediation of pinocytosis in cultured arterial smooth muscle and endothelial cells by platelet-derived growth factor. J Cell Biol 1978, 79:663–671
41. Kazlauskas A, DiCorleto PE: Cultured endothelial cells do not respond to a platelet-derived growth factor-like protein in an autocrine manner. Biochim Biophys Acta 1985, 846:405–412
42. Bowen-Pope DF, Ross R: Platelet-derived growth factor. II. Specific binding to cultured cells. J Biol Chem 1982, 257:5161–5171
43. Narayanan SA, Page RC: Biosynthesis and regulation of type V collagen in diploid human fibroblasts. J Biol Chem 1983, 258:11694–11699
44. Chua CC, Geiman DE, Keller GH, Ladda RL: Induction of collagenase secretion in human fibroblast cultures by growth promoting factors. J Biol Chem 1985, 260:5213–5216
45. Bauer EA, Cooper TW, Huang JS, Altman J, Deuel TF: Stimulation of *in vitro* human skin collagenase expression by platelet-derived growth factor. Proc Natl Acad Sci USA 1985, 82:4132–4136
46. Laiho M, Saksela O, Keski-Oja J: Transforming growth factor β alters plasminogen activator activity in human skin fibroblasts. Exp Cell Res 1986, 164:399–407
47. Heimark RL, Twardzik DR, Schwartz SM: Inhibition of endothelial regeneration by type-beta transforming growth factor from platelets. Science 1986, 233:1078–1080
48. Ignotz RA, Massague J: Transforming growth factor-β stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. J Biol Chem 1986, 261:4337–4345
49. Laiho M, Saksela O, Andreasen PA, Keski-Oja J: Enhanced production and extracellular deposition of the endothelial-type plasminogen activator inhibitor in cultured human lung fibroblasts by transforming growth factor-β. J Cell Biol 1986, 103:2403–2410
50. Edwards DR, Murphy G, Reynolds JJ, Whitham SE, Docherty AJP, Angel P, Heath JK: Transforming growth factor beta modulates the expression of collagenase and metalloproteinase inhibitor. EMBO J 1987, 6:1899–1904
51. Senior RM, Huang SS, Griffin GL, Huang JS: Brain-derived growth factor is a chemoattractant for fibroblasts and astroglial cells. Biochem Biophys Res Commun 1986, 141:67–72

52. Gospodarowicz D, Moran JS, Braun DL: Control of proliferation of bovine vascular endothelial cells. J Cell Physiol 1977, 91:377–386
53. Gospodarowicz D, Moran JS: Mitogenic effect of fibroblast growth factor on early passage cultures of human and murine fibroblasts. J Cell Biol 1975, 66:451–457
54. Montesano R, Vassalli J-D, Baird A, Guillemin R, Orci L: Basic fibroblast growth factor induces angiogenesis *in vitro*. Proc Natl Acad Sci USA 1986, 83:7297–7301
55. Schreiber AB, Winkler ME, Derynck R: Transforming growth factor-α: A more potent angiogenic mediator than epidermal growth factor. Science 1986, 232:1250–1253
56. Knighton DR, Fiegel VD, Austin LL, Ciresi KF, Butler EL: Classification and treatment of chronic nonhealing wounds: Successful treatment with autologous platelet-derived wound healing factors. Ann Surg 1986, 204:322–330
57. Grotendorst GR, Martin GR, Pencev D, Sodek J, Harvey AK: Stimulation of granulation tissue formation by platelet-derived growth factor in normal and diabetic rats. J Clin Invest 1985, 76:2323–2329
58. Lawrence WT, Sporn MB, Gorschboth C, Norton JA, Grotendorst GR: The reversal of an Adriamycin induced healing impairment with chemoattractants and growth factors. Ann Surg 1986, 203:142–147
59. Lobb R, Sasse J, Sullivan R, Shing Y, D'Amore P, Jacobs J, Klagsbrun M: Purification and characterization of heparin-binding endothelial cell growth factors. J Biol Chem 1986, 261:1924–1928
60. Davidson JM, Klagsburn M, Hill KE, Buckley A, Sullivan R, Brewer PS, Woodward SC: Accelerated wound repair, cell proliferation, and collagen accumulation are produced by a cartilage-derived growth factor. J Cell Biol 1985, 100:1219–1227
61. Baird A, Esch F, Gospodarowicz D, Guillemin R: Retina- and eye-derived endothelial cell growth factors: Partial molecular characterization and identity with acidic and basic fibroblast growth factors. Biochemistry 1985, 24:7855–7860
62. Fourtanier AY, Courty J, Muller E, Courtois Y, Prunieras M, Barritault D: Eye-derived growth factor isolated from bovine retina and used for epidermal wound healing *in vivo*. J Invest Dermatol 1986, 87:76–80
63. Sporn MB, Roberts AB, Shull JH, Smith JM, Ward JM, Sodek J: Polypeptide transforming growth factors isolated from bovine sources and used for wound healing *in vivo*. Science 1983, 219:1329–1331
64. Niall M, Ryan GB, O'Brien BM: The effect of epidermal growth factor on wound healing in mice. J Surg Res 1982, 33:164–169
65. Hiramatsu M, Hatakeyama K, Minami N, Kumegawa M: Increase in collagen synthesis of cotton pellet granuloma in rats by epidermal growth factor. Japan J Pharmacol 1982, 32:198–201
66. Buckley A, Davidson JM, Kamerath CD, Wolt TB, Woodward SC: Sustained release of epidermal growth factor accelerates wound repair. Proc Natl Acad Sci USA 1985, 82:7340–7344

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